Multivalent Smallpox DNA Vaccine Delivered by Intradermal Electroporation Drives Protective Immunity in Nonhuman Primates Against Lethal Monkeypox Challenge

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The threat of a smallpox-based bioterrorist event or a human monkeypox outbreak has heightened the importance of new, safe vaccine approaches for these pathogens to complement older poxviral vaccine platforms. As poxviruses are large, complex viruses, they present technological challenges for simple recombinant vaccine development where a multicomponent mixtures of vaccine antigens are likely important in protection. We report that a synthetic, multivalent, highly concentrated, DNA vaccine delivered by a minimally invasive, novel skin electroporation microarray can drive polyvalent immunity in macaques, and offers protection from a highly pathogenic monkeypox challenge. Such a diverse, high-titer antibody response produced against 8 different DNA-encoded antigens delivered simultaneously in microvolumes has not been previously described. These studies represent a significant improvement in the efficiency of the DNA vaccine platform, resulting in immune responses that mimic live viral infections, and would likely have relevance for vaccine design against complex human and animal pathogens.

Smallpox infection is an exceptionally contagious and highly lethal pathogen, with a lethality rate of >33% for some forms of smallpox. After its eradication by a vaccination campaign using smallpox vaccine (Dryvax, Wyeth Laboratories), a live attenuated vaccine, there was a low level of interest in smallpox vaccination by the general public or the scientific community. However, after 11 September 2001, significant concerns over possible bioterrorism with this agent or an engineered smallpox agent have reemerged. In addition, monkeypox, a related infectious pathogen with significant mortality in humans, is an emerging concern [1].

Despite the success of the Dryvax vaccine, there were numerous vaccine safety concerns relating to changing global health demographics over the last half-century. Accordingly, a less virulent stock consisting of modified vaccinia virus Ankara (MVA) stock has been developed and has shown improved safety in phases I and II clinical trials [2, 3]. Although MVA is much less virulent than Dryvax, it remains clear that an alternative nonlive approach could be of additional security for specific compromised populations or in situations where unintended spread is a particular concern.

In this regard, DNA vaccines are considered a safe vaccination platform. However, a number of obstacles must be overcome to generate an immune-potent DNA vaccine for smallpox or monkeypox. Historically, DNA vaccination has been less immunogenic in nonhuman primate studies,

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as well as in human clinical trials, compared with live viral approaches [4]. In addition, previous DNA, as well as recombinant protein, vaccine studies have used a limited number of antigens [5–9] due to technological limitations. However, smallpox is a highly complex DNA virus that encodes over 200 genes and has two infectious forms, the mature virion (MV) and the enveloped virion (EV), each with its own unique set of membrane proteins [10]. Given the complex antigenic nature of this virus, we have focused on a assembling a multiantigen cocktail in an attempt to provide adequate antigenic coverage for both infectious forms of the virus. Our plasmid cocktail contains MV neutralizing antibody targets A27 [11, 12], F9 [13], H3 [14, 15], and L1 [16]. Additionally, we incorporated EV antigens A33, A56 [17], and B5. Although B5 [11] is the only EV neutralizing target, A33 has been shown to enhance the protection conferred by L1 immunization in murine challenge studies [18, 19]. The core antigen A4 was also used to enhance the effect of cytotoxic T lymphocytes in a monkeypox challenge model.

A number of studies have demonstrated the importance of neutralizing antibodies in the control of poxviral infections [11, 20, 21]. While DNA vaccines have been shown to induce antibodies in a number of small animal studies, they have been largely used to induce cellular immune responses [22]. To address this issue, we compared the delivery of antigens by the intradermal (ID) route, a route that has been associated with the development of predominantly T_H2 responses [23], and the traditional intramuscular (IM) route.

To test the efficacy of these strategies, we immunized a total of 14 cynomolgus macaques with our multivalent smallpox DNA vaccine either by the ID or IM route. We monitored the magnitude, quality, and efficacy of the vaccine-induced response to provide protection during a lethal monkeypox Zaire 79 challenge. We report that the vaccine was able to elicit both a broad and robust binding and neutralizing antibody response similar to that induced by Dryvax. Potent cellular immunity was also observed. The combination of immune responses was able to dramatically impact a lethal poxviral challenge in macaques. These findings have important implications for the use of DNA vaccine technology against emerging infectious diseases.

METHODS

Animals. A total of 14 cynomolgus macaques (4 controls, 4 IM immunized, 6 ID immunized) were housed and cared for by Southern Research Institute (Birmingham, Alabama). The experimental design was in accordance with the guidelines set forth by IACUC at the Southern Research Institute, the Guide for the Care and Use of Laboratory Animals, 7th Edition, and the USDA through the Animal Welfare Act (Public Law 99–198).

Cloning of the DNA Expression Constructs. The VACV Western Reserve (WR) Strain genes, A4L, A27L, A33R, A56R, B5R, F9L, H3L, and L1R, were chemically synthesized, human codon-optimized, and modified to contain a Kozak consensus sequence and IgE leader sequence at the 5' end, and an influenza hemagglutinin epitope tag at the 3' end of the DNA sequence. Each of these modified gene cassettes were cloned into the eukaryotic expression plasmid, pVAX1 (Invitrogen, Carlsbad, California) by GENEART (Burlingame, California). After cloning, all antigens were confirmed by sequencing.

Vaccine Preparation. Plasmids were manufactured to high concentrations and purified using the manufacturing procedure described by Hebel et al [24] in US patent 7238522 with modifications. All plasmid preparations were formulated and prepared with 1% weight/weight with high performance liquid chromatography purified low-molecular-weight poly-L-glutamate in sterile water, as previously described [24]. All plasmids (pGX4001 to pGX4008) were combined to make a single vaccine preparation consisting of 125 μg of each plasmid in a total volume of 0.1 mL for the ID or 0.5 mL for the IM administration.

Immunization. Animals were anesthetized intramuscularly with ketamine HCL (10–30 mg/kg). The vaccine was administered to each thigh (one injection site per thigh per vaccination) and delivered either by the ID route or IM route. Immunization via IM was in the semimembranosus muscle in combination with electroporation (EP) using the CELLECTRA 2000 device (Inovio Pharmaceuticals Blue Bell, Pennsylvania). Immediately following the injection, a 2 × 2 pulse sequence at 0.2 A constant current, 52 ms pulse length with 1 s between pulses was applied for ID administration, and 3 pulses at 0.5 A constant current with 52 ms pulse length with 1s between pulses was applied for IM administration. Immunizations were performed at days 0, 28, and 56.

Preparation of the Antigens for Antigen-Specific ELISA. The open reading frames of each antigen were PCR amplified from VACV WR and cloned into the pEt219a(+) vector (EMD Chemicals, San Diego, CA). The 3′-end oligonucleotide was designed to allow fusion with the 6x-histidine tag present in pEt219a(+). Proteins were purified using standard nickel column purification methods by Abgent (San Diego, CA).

Antigen-Specific ELISA. To determine the IgG antibody responses, ELISAs were performed as previously described [25]. Antibody responses to the 8 pox antigens are reported as endpoint titers, which are expressed as the reciprocal of the highest serum dilution yielding a positive reactivity greater than twofold above a negative control serum.

Synthetic Peptides. Total antigen peptide libraries were synthesized based on VACV WR. All peptides were 15-mers overlapping by either 9 amino acids (A4L and A27L), 11 amino acids (A33R, A56R, F9L, H3L, and L1R), or 6 amino acids (B5R). The peptide libraries were prepared by Invitrogen (A4L and A27L) and GenScript (Piscataway, NJ). Libraries were prepared as the corresponding peptide pool at a concentration of 10 mg/ml in dimethyl sulfoxide.

IFN-γ ELISPOT Assay. The nonhuman primate ELISPOT assays were performed as previously described [26].

Antigen-specific responses were determined by subtracting the number of spots in the negative control wells from the wells containing peptides. Results are shown as the mean value (spots/million splenocytes) obtained for triplicate wells.

Carboxyfluorescein Succinimidyl Ester (CFSE) Proliferation and Intracellular Cytokine Staining (ICS). CFSE and ICS analyses were performed on freshly isolated peripheral blood mononuclear cells (PBMCs) following the third immunization, as previously described [25]. The data are shown after background correction.

Monkeypox Virus challenge. The Zaire strain, V79-I-005 (monkeypox virus [MPV] Master Seed NR-523), of monkeypox virus was obtained from the NIH Biodefense and Emerging Infections Research Resources Repository. Cynomolgus macaques were intravenously infused with 2×10^7 PFU of MPV NR-523 into the saphenous vein. In order to confirm the actual delivered dose, the challenge inoculum was back-tittered on Vero E6 cells using a standard plaque assay technique.

Real-Time PCR to Detect MPV Genomes. DNA was extracted from frozen blood samples and viral genomes were detected by real-time PCR as previously described [27].

Measurement of MPV-Neutralizing Antibody. Serum from the monkeys were collected throughout the duration of the study, heat-inactivated (56°C for 30 min), and evaluated for the presence of MPV neutralizing antibodies using classical plaque reduction neutralization tests as previously described [28].

Plasma samples from three ID and three pVAX1 animals were collected on Day 91 prechallenge, and serum samples from three Dryvax-immunized rhesus macaques (generously provided by Dr. Rama Rao), which had been collected one month following their immunization, were analyzed in a MPV neutralizing antibody assay (Slifka laboratory, OHSU, Beaverton, OR). Serial twofold dilutions of heat-inactivated serum were incubated with MPV (Zaire strain, ~64 plaque-forming units) for 2 h at 37°C before plating the mixture on Vero cell monolayers in 6-well plates. After 1 h, the cells were overlaid with 0.5% agarose and incubated for 4 days to allow for plaque formation. Monolayers were fixed with 75% methanol/25% acetic acid, the agarose removed, and the monolayer stained with 0.1% crystal violet in PBS containing 0.2% formaldehyde. The neutralization titer 50% was defined as the serum dilution resulting in a 50% reduction of plaques, and was calculated by log–log transformation of the linear portion of the curve. Logarithmic transformation of the data was used to calculate the titer, and conversion was performed on the final values.

Statistical Analysis. Data are represented as the mean ± standard error of the mean for comparisons of neutralizing antibody titers and viral loads, one-way ANOVA tests (Kruskal-Wallis) were performed using GraphPad Prism software. For $P < .05$, these P values were considered significant and reported. The Spearman Rank correlation (nonparametric) test was used to evaluate the correlation between neutralizing antibody titers and lesion counts.

RESULTS

Study Design

Two groups of cynomolgus macaques were immunized 3 times, one month apart, with a multivalent DNA vaccine comprising 8 vaccinia virus (VV) Western Reserve strain genes: A4L, A27L, A33R, A56R, B5R, F9L, H3L, and L1R. One group of macaques ($n = 6$) received the dose of DNA, 250 μg/antigen, divided into two injections delivered into the quadricep by the ID route. Likewise, a second group of macaques ($n = 4$) was immunized by the IM route. Finally, a group ($n = 4$) of animals were immunized with the empty vector (pVAX1) and used as a negative control. One month following the third immunization, the animals were challenged with a lethal dose of the Zaire 79 strain of the MPV.

Induction of Broad Cellular Immune Responses

We first sought to assess the efficacy of our multivalent vaccine on the induction of cellular immune responses by the standard, quantitative IFNγ ELISPOT assay (Figure 1). After three immunizations, the total vaccine response in all of the groups were between 900 and 1,775 SFU/million PBMCs, with most vaccinated animals having a positive IFNγ response to 6 of the 8 antigens. Immunization via the IM route resulted in higher IFNγ responses than immunization by the ID route, exhibiting a robust 1775 ± 241 SFU/million PBMCs.

We further characterized the immune phenotype of the resulting cellular response by intracellular cytokine staining for IFNγ, IL-2, and TNF-α production, as well as CD107a as a surrogate marker for degranulation. The overall magnitude of functional responses was higher in the $CD4^+$ T cell compartment than the $CD8^+$ T cell compartment. The IM group had the highest $CD4^+$ T cell response with an average magnitude of 0.4% ± 0.11% of $CD4^+$ T cells producing a functional response (Figure 2A). The ID-immunized animals exhibited a slightly lower average response (0.28% ± 0.10%). The $CD8^+$ responses were similar between the ID and IM immunized groups (0.26% ± 0.09% and 0.21% ± 0.17%, respectively) (Figure 2B).

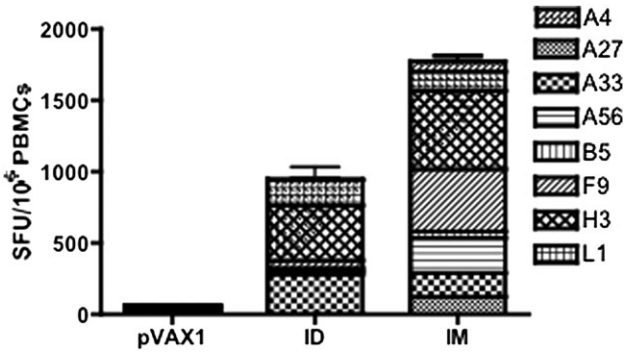

Figure 1. IFN-γ responses to VACV antigens following immunization. PBMCs were isolated 2 weeks following the third immunization, and antigen-specific IFN-γ responses were assessed by quantitative ELISPOT. Results are shown as stacked group mean responses ± standard error of the mean.

Using Boolean gating, we examined the polyfunctional nature of the cellular response. In general, the responding animals generated a monofuctional response, with CD107a being the predominantly observed function.

Another parameter of the cellular immune response is the proliferative capacity of the vaccine-induced T cell response. We isolated PBMCs following the third immunization and stimulated them ex vivo and assessed proliferation by CFSE dilution. $CD4^+$ T cell proliferation was highest in the ID group (11.7% ± 7.0%) (Figure 3A). The IM groups exhibited a lower $CD4^+$ T cell response (1.48% ± 1.12%). Similar results were seen in the $CD8^+$ T cell compartment, with the ID group exhibiting the highest response (6.7% ± 5.4%) by several-fold (Figure 3B).

Induction of Antigen-Specific and Neutralizing Antibody Responses

Antibody responses to the virus have been reported to be important for protection from a poxviral challenge. We assessed the ability of the multivalent DNA vaccines to induce antibody responses against recombinant VACV proteins. Most antigens in the multivalent vaccine elicited an antibody response to varying degrees irrespective of the route of vaccination (Table 1). In general, delivery by the ID route fared better than the IM delivery, producing a detectable response to the L1 antigen.

We next examined the neutralizing antibody response by an in vitro PRNT assay. MPV neutralizing antibody titers were measured prior to challenge (Figure 4A). As expected, serum samples collected from pVAX1-treated animals were unable to neutralize the virus. The ID group exhibited the largest induction of neutralizing antibodies, with an average endpoint titer of 267 ± 159. As observed with the previous ELISA data, the IM group exhibited lower levels of neutralizing antibodies (39 ± 28).

In a separate experiment, we compared the prechallenge neutralizing antibody titer of 3 animals in the ID group to 3 serum samples from Dryvax-immunized macaques (Figure 4B). The ID animals exhibited an average titer of 330 ± 126, which was fairly comparable to the Dryvax-immunized control animals that had an average neutralizing antibody titer of 407 ± 263.

Reduction in the Level of Viremia and the Boosting of Neutralizing Antibody Responses Following Lethal Monkeypox Challenge

To evaluate the efficacy of the vaccine-induced response, animals were challenged with a lethal dose, 2×10^7 PFU, of the Zaire NR-523 strain of MPV by intravenous (IV) administration. This challenge stock was derived from a viral stock collected from a lethally infected human in Zaire [29], supporting its importance in human infection and disease. All macaques demonstrated an MPV infection with peak viremia levels

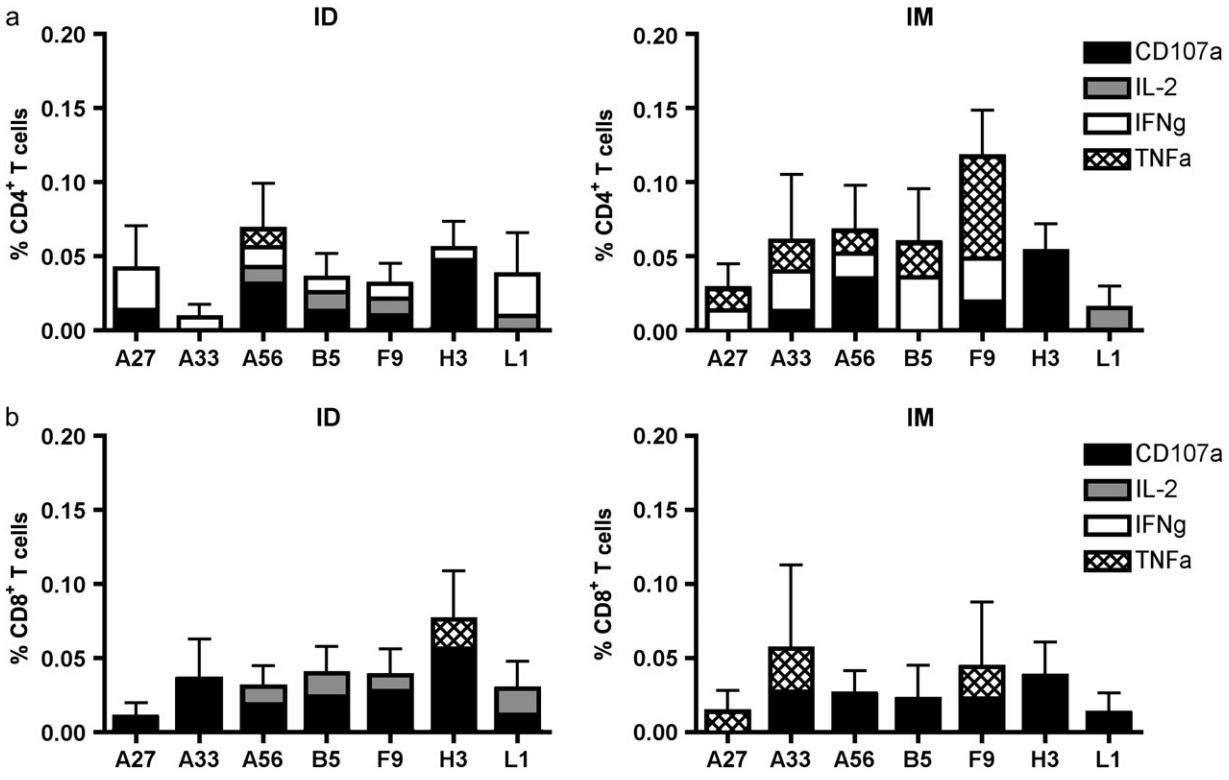

Figure 2. Antigen-specific T cell function following immunization. PBMCs isolated 2 weeks after the third immunization were stimulated in vitro with total peptide pool mixes for 5 h. Cells were stained for intracellular production of IFN-γ, TNF-α, and IL-2, and degranulated by CD107a. The functional phenotype was assessed for $CD4^+$ (A) and $CD8^+$ (B) T cells. The stacked bar graphs depict the average magnitude of all functional responses for each immunization group.

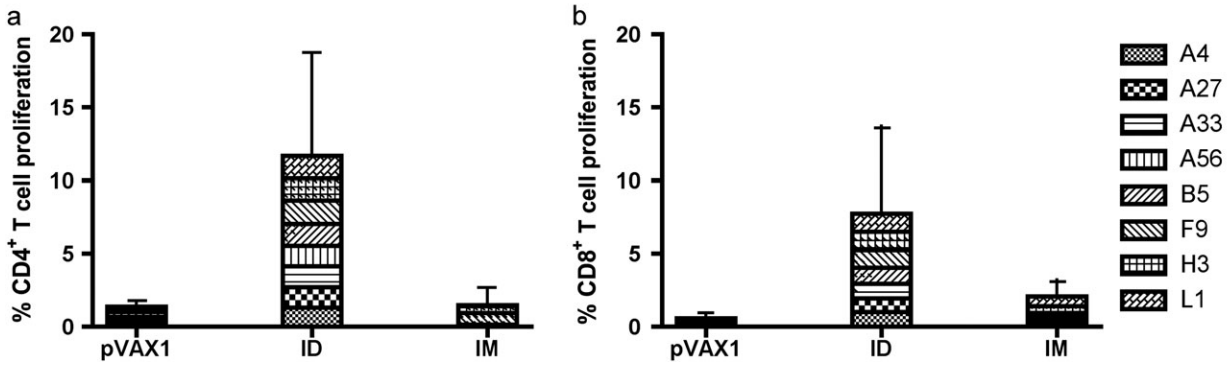

Figure 3. Proliferative capacity of $CD4^+$ and $CD8^+$ T cells. Fresh PBMCs isolated 4 weeks following the third immunization were stained with CFSE and stimulated with antigen-specific peptides *in vitro* for 5 days to determine the proliferative capacity of antigen-specific (A) $CD4^+$ and (B) $CD8^+$ T cells. Results are shown as stacked group mean responses ± standard error of the mean.

developing 6 to 9 days postchallenge (Figure 5A). The multivalent vaccine provided protection irrespective of route of administration, with all animals surviving the challenge. In contrast, the pVAX1 vector-control animals developed typical symptoms of monkeypox disease, with 3 of the 4 animals being euthanized by day 15 postchallenge due to severity of the disease. The level of viremia peaked in pVAX1-vaccinated animals at 8.5 log RNA copies/ml 12 days postchallenge. In contrast, the animals in the ID group exhibited a significant reduction in the level of viremia by >3 logs on day 12 ($P = .01$, Kruskal-Wallis) with undetectable levels (<5,000 copies/mL) of the virus observed at the end of the study. Thus, these findings demonstrate the protective efficacy of this DNA vaccine formulation in generating protective immunity capable of controlling MPV viremia.

We also monitored the anamnestic neutralizing antibody response following challenge. Sera samples collected 6 days following challenge were assessed for neutralizing antibody titers (Figure 5B). All of the vaccinated groups mounted robust anamnestic responses (range 4,820–7,255) compared with the pVAX1-immunized group (737 ± 366).

Table 1. Antigen-specific antibody titers

	Group		
Antigen	ID	IM	pVAX1
A4	2,667 ± 1,667	<100	<100
A27	6,709 ± 2,082	7,500 ± 2,500	<100
A33	7,500 ± 1,708	10,000 ± 0	<100
A56	11,667 ± 4,595	9,063 ± 5,756	<100
B5	3,334 ± 2,108	1,251 ± 721	<100
F9	7,500 ± 2,041	2,501 ± 2,500	<100
H3	4,167 ± 2,007	<100	<100
L1	1,668 ± 1,667	<100	<100

NOTE. Data are shown as group mean end point titers ± standard error of the mean.

Multivalent DNA Vaccine Protects Nonhuman Primates from Clinical Symptoms of Severe Monkeypox Disease

In addition to mortality and viral loads following monkeypox challenge, other clinical signs of monkeypox disease were monitored. Weight loss and increase in body temperature were used as indicators of morbidity and were measured following viral challenge. The pVAX1-treated animals experienced sustained weight loss during the acute phase of the infection. The largest weight loss was observed on day 12 postchallenge when pox lesions and viremia peaked (Figure 6A). On day 12 of the challenge, the average weight loss was 8.73%. The one surviving pVAX1-immunized macaque eventually regained weight, but not until day 21 postchallenge. In contrast, vaccinated animals did not experience significant weight loss postchallenge.

For the pVAX1-treated animals, an increase in body temperature was observed up to day 9 postchallenge (Figure 6B). The pVAX1-treated animals exhibited elevated body temperatures compared with the vaccinated groups. The average temperature in vaccinated animals was maintained within normal body temperature (99–102°F) throughout the challenge.

Another parameter that was measured was the development of lesions following challenge (Figure 6C). The vaccinated groups had very low peak lesion counts (ID: 133 ± 37; IM: 175 ± 26) that negatively correlated with prechallenge neutralizing antibody titers (Spearman's rank correlation, −.528; $P = .008$) (Figure 6D). Only one macaque from the control group survived the challenge but with lesions still present at the end of the observation period (day 27 postchallenge).

DISCUSSION

A protective DNA vaccine would represent an additional alternative to the live viral vaccines currently used for the prevention of smallpox infection, which could become an important tool in poxviral control [30]. Furthermore, there are growing concerns regarding lethal monkeypox spreading to human populations [1]. DNA vaccines have a number of conceptual advantages as

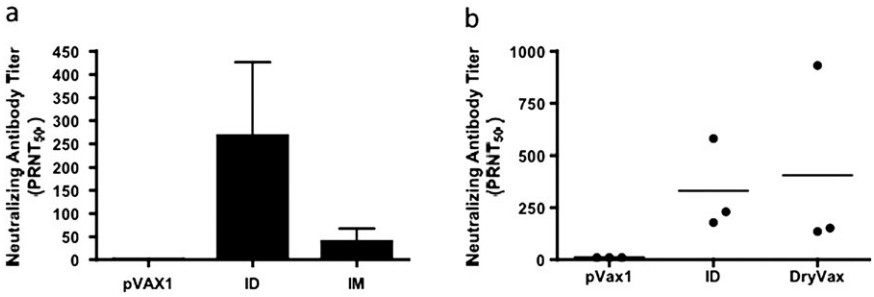

Figure 4. Prechallenge neutralizing antibody titers against monkeypox virus. Serum samples were collected from animals on day 91 (prechallenge) and evaluated for the presence of neutralizing antibodies against the Zaire 79 strain of monkeypox virus by plaque reduction neutralization tests (PRNT) (A). In a separate experiment, plasma samples from three ID and three pVAX1 animals collected on day 91 prechallenge and serum samples from three Dryvax immunized rhesus macaques collected 1 month following immunization were used in a monkeypox neutralizing antibody assay (B). Titers represent the reciprocal of the highest dilution, resulting in a 50% reduction in the number of plaques. Mean values ± standard error of the mean are shown.

a vaccine in this regard, such as safety and simplicity of manufacturing, compared with their live viral counterparts. However, until recently, IM injection of plasmid DNA has failed to generate substantial protective immune responses in large animal models as well as in human clinical trials [4].

This study assessed the tolerability, immunogenicity, and efficacy of a multivalent DNA smallpox vaccine candidate. The antigens selected have been shown to individually and in combination yield both a robust antibody and cellular immune response, and have provided some protection, particularly in small animal models [9, 18, 31–34]. We observed that this high-formulation DNA vaccine preparation delivered either via the ID or IM route followed by EP provided protection against a lethal challenge of MPV in nonhuman primates.

Following immunization, we observed strong IFN-γ responses. While the IM group exhibited higher total IFN-γ responses, the ID group exhibited better $CD4^+$ and $CD8^+$ T cell proliferation. Polyfunctional analysis demonstrated higher overall $CD4^+$ T cell responses induced by ID vaccination, while IM vaccination yielded higher responses in the $CD8^+$ T cell compartment. However, regardless of the immunization route, both $CD4^+$ and $CD8^+$ T cells induced were predominantly monofunctional with a displayed association of a killing phenotype. However, this is most likely not due to the method of vaccination, since a previous study with IM EP of HIV antigens has reported an induction of a four-functional response [25]. Thus, the functional profile of the vaccine-induced immune response observed in this study is likely an antigen-related phenomenon.

Numerous studies have demonstrated the importance of antibody responses in the protection from poxviral infection [11, 20, 21]. Induction of useful antibody responses in nonhuman primates and humans has been a particular weakness of DNA vaccines. In this study, we observed the induction of high-titer antibody responses to 8 of 8 antigens in the ID group, and 5 of 8 antigens in the IM group. L1 responses, an important MV neutralization target [16], were among the lowest responses in the ID group and were not detected in the IM group. However, this result is not unexpected, as a previous study using recombinant protein immunization reported undetectable antibody responses to L1 protein unless supplemented with adjuvants [35]. In addition, a recent study (completed after the present work was initiated) reported that modification of an L1 construct could yield better antibody responses [36, 37].

In addition to higher-binding antibody responses, the ID group had a mean neutralizing antibody titer that was ~5-fold higher than the IM immunization group. The titers induced in

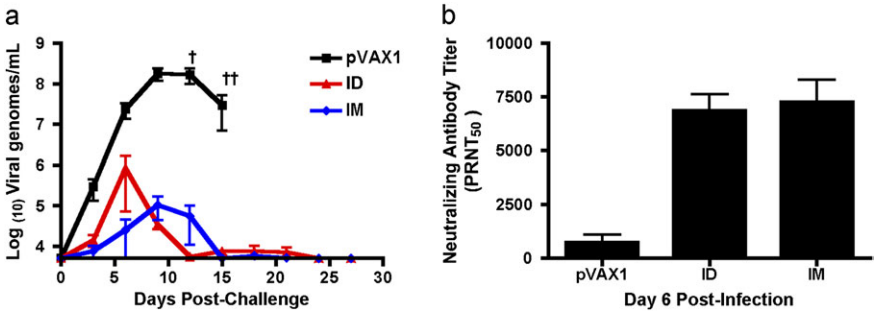

Figure 5. Viral loads and neutralizing antibody titers following lethal IV challenge with monkeypox Zaire 79. The number of monkeypox virus genomes per milliliter of blood was determined by quantitative TaqMan 3'-minor groove binder PCR (A). The lower limit of detection was 5,000 genomes/mL of blood. Anamnestic neutralizing antibody responses were assessed 6 days postchallenge by PRNT (B). Mean values ± standard error of the mean are shown.

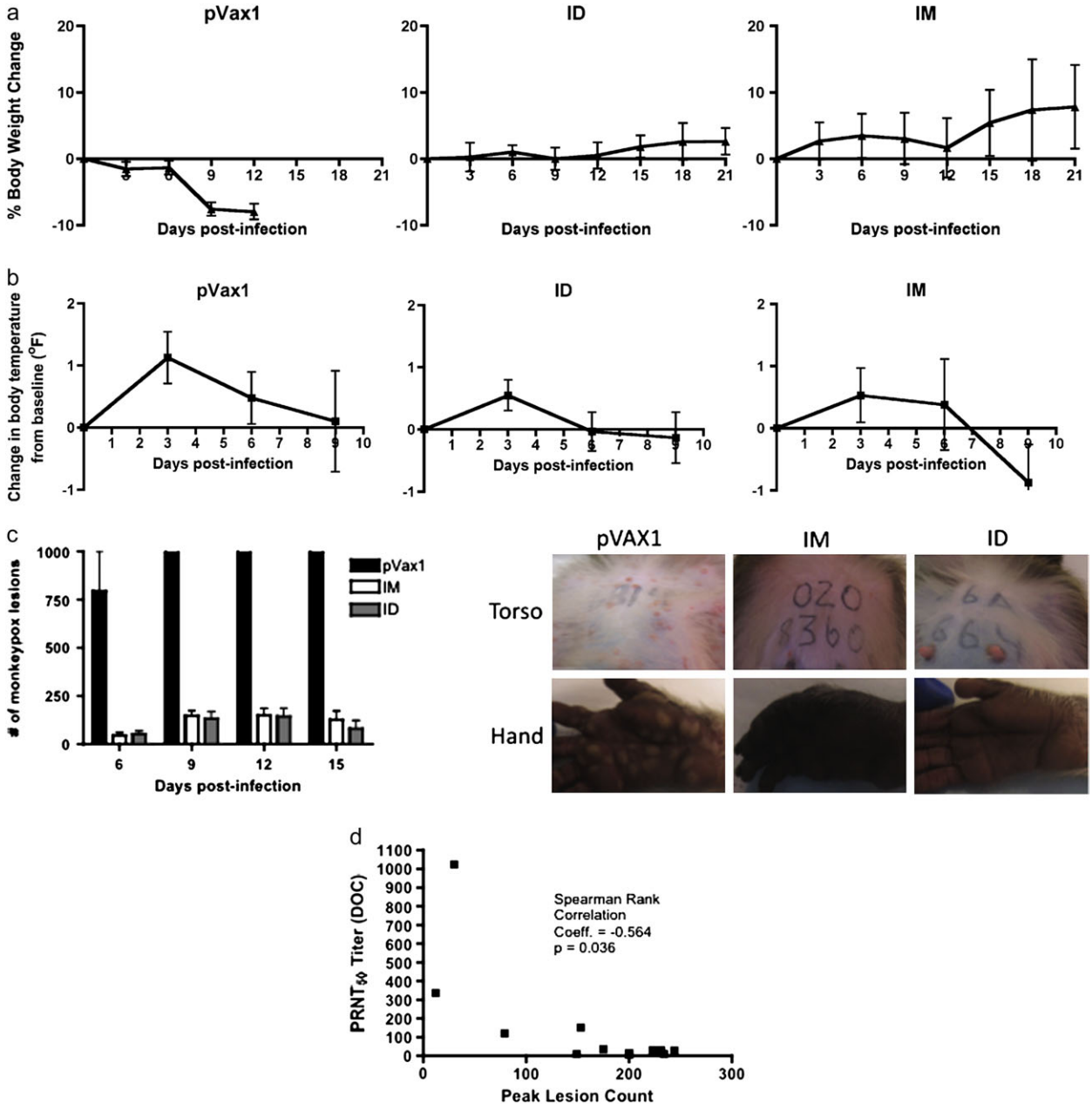

Figure 6. Clinical signs of monkeypox disease following intravenous challenge. In addition to viral loads, changes in body weight (A), body temperature (B), and the development of monkeypox lesions (C) were monitored to assess the severity of monkeypox disease. Representative animals from the pVAX1, IM, and ID groups at day 12 postchallenge are shown (D). Spearman rank correlation of MPV neutralizing antibody titers and maximum number of pock lesions are indicated. Body temperature and weight results are shown as percent change compared with prechallenge baseline values. Group mean ± standard error of the mean are shown.

the ID-vaccinated animals were comparable to responses seen in rhesus macaques and humans following Dryvax immunization [38]. Furthermore, these responses negatively correlated with peak lesion counts and were further boosted following challenge, indicating the development of robust memory B cell populations.

These results are important in that they demonstrate the collective ability to continually improve the DNA vaccine platform. The concentrated formulations delivered by the IM or ID EP routes allowed for the development of high-titer protective antibody responses after immunizations with doses that are a log lower than traditional DNA immunization. Furthermore, the efficacy of the ID group is encouraging, as it further enhances the tolerability of the EP platform. Further study of this combination of technologies to enhance DNA vaccine potency in vaccine arenas where antibody responses are important are worthy of further study in areas such as bioterrorism.

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References

1. Ladnyj ID, Ziegler P, Kima E. A human infection caused by monkeypox virus in Basankusu Territory, Democratic Republic of the Congo. *Bull World Health Organ.* **1972**; 46:593–7.
2. Parrino J, McCurdy LH, Larkin BD, et al. Safety, immunogenicity and efficacy of modified vaccinia Ankara (MVA) against Dryvax challenge in vaccinia-naive and vaccinia-immune individuals. *Vaccine.* **2007**; 25:1513–25.
3. von Krempelhuber A, Vollmar J, Pokorny R, et al. A randomized, double-blind, dose-finding Phase II study to evaluate immunogenicity and safety of the third generation smallpox vaccine candidate IMVAMUNE. *Vaccine.* **2010**; 28:1209–16.
4. Hokey DA, Weiner DB. DNA vaccines for HIV: challenges and opportunities. *Springer Semin Immunopathol.* **2006**; 28:267–79.
5. Hooper JW, Thompson E, Wilhelmsen C, et al. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. *J Virol.* **2004**; 78:4433–43.
6. Berhanu A, Wilson RL, Kirkwood-Watts DL, et al. Vaccination of BALB/c mice with Escherichia coli-expressed vaccinia virus proteins A27L, B5R, and D8L protects mice from lethal vaccinia virus challenge. *J Virol.* **2008**; 82:3517–29.
7. Earl PL, Americo JL, Wyatt LS, et al. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. *Nature.* **2004**; 428:182–5.
8. Heraud JM, Edghill-Smith Y, Ayala V, et al. Subunit recombinant vaccine protects against monkeypox. *J Immunol.* **2006**; 177: 2552–64.
9. Hooper JW, Custer DM, Thompson E. Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. *Virology.* **2003**; 306:181–95.
10. Vanderplasschen A, Hollinshead M, Smith GL. Intracellular and extracellular vaccinia virions enter cells by different mechanisms. *J Gen Virol.* **1998**; 79(pt 4);877–87.
11. Putz MM, Midgley CM, Law M, Smith GL. Quantification of antibody responses against multiple antigens of the two infectious forms of Vaccinia virus provides a benchmark for smallpox vaccination. *Nat Med.* **2006**; 12:1310–5.
12. Rodriguez JF, Janeczko R, Esteban M. Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. *J Virol.* **1985**; 56:482–8.
13. Brown E, Senkevich TG, Moss B. Vaccinia virus F9 virion membrane protein is required for entry but not virus assembly, in contrast to the related L1 protein. *J Virol.* **2006**; 80:9455–64.
14. Lin CL, Chung CS, Heine HG, Chang W. Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. *J Virol.* **2000**; 74:3353–65.
15. Davies DH, McCausland MM, Valdez C, et al. Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. *J Virol.* **2005**; 79:11724–33.
16. Wolffe EJ, Vijaya S, Moss B. A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. *Virology.* **1995**; 211:53–63.
17. Seki M, Oie M, Ichihashi Y, Shida H. Hemadsorption and fusion inhibition activities of hemagglutinin analyzed by vaccinia virus mutants. *Virology.* **1990**; 175:372–84.
18. Hooper JW, Custer DM, Schmaljohn CS, Schmaljohn AL. DNA vaccination with vaccinia virus L1R and A33R genes protects mice against a lethal poxvirus challenge. *Virology.* **2000**; 266:329–39.
19. Galmiche MC, Goenaga J, Wittek R, Rindisbacher L. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. *Virology.* **1999**; 254:71–80.
20. Edghill-Smith Y, Golding H, Manischewitz J, et al. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. *Nat Med.* **2005**; 11:740–7.
21. Panchanathan V, Chaudhri G, Karupiah G. Protective immunity against secondary poxvirus infection is dependent on antibody but not on CD4 or CD8 T-cell function. *J Virol.* **2006**; 80:6333–8.
22. Cristillo AD, Weiss D, Hudacik L, et al. Persistent antibody and T cell responses induced by HIV-1 DNA vaccine delivered by electroporation. *Biochem Biophys Res Commun.* **2008**; 366:29–35.
23. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol.* **1997**; 158:2278–84.
24. Hebel H, Attra H, Khan A, Draghia-Akli R. Successful parallel development and integration of a plasmid-based biologic, container/closure system and electrokinetic delivery device. *Vaccine.* **2006**; 24:4607–14.
25. Hirao LA, Wu L, Khan AS, et al. Combined effects of IL-12 and electroporation enhances the potency of DNA vaccination in macaques. *Vaccine.* **2008**; 26:3112–20.
26. Boyer JD, Robinson TM, Kutzler MA, et al. SIV DNA vaccine coadministered with IL-12 expression plasmid enhances CD8 SIV cellular immune responses in cynomolgus macaques. *J Med Primatol.* **2005**; 34:262–70.
27. Hooper JW, Ferro AM, Golden JW, et al. Molecular smallpox vaccine delivered by alphavirus replicons elicits protective immunity in mice and non-human primates. *Vaccine.* **2009**; 28:494–511.
28. Nigam P, Earl PL, Americo JL, et al. DNA/MVA HIV-1/AIDS vaccine elicits long-lived vaccinia virus-specific immunity and confers protection against a lethal monkeypox challenge. *Virology.* **2007**; 366:73–83.
29. Jordan R, Goff A, Frimm A, et al. ST-246(R) antiviral efficacy in a nonhuman primate monkeypox model: determination of the minimal effective dose and human dose justification. *Antimicrob Agents Chemother.* **2009**; 53(5):1817–22.
30. Andrew WA. New generation smallpox vaccines: a review of preclinical and clinical data. *Rev Med Virol.* **2008**; 18:217–31.
31. Hooper JW, Golden JW, Ferro AM, King AD. Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge. *Vaccine.* **2007**; 25:1814–23.
32. Otero M, Calarota SA, Dai A, De Groot AS, Boyer JD, Weiner DB. Efficacy of novel plasmid DNA encoding vaccinia antigens in improving current smallpox vaccination strategy. *Vaccine.* **2006**; 24:4461–70.
33. Demkowicz WE, Maa JS, Esteban M. Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. *J Virol.* **1992**; 66:386–98.
34. Sivapalasingam S, Kennedy JS, Borkowsky W, et al. Immunological memory after exposure to variola virus, monkeypox virus, and vaccinia virus. *J Infect Dis.* **2007**; 195:1151–9.
35. Fogg CN, Americo JL, Lustig S, et al. Adjuvant-enhanced antibody responses to recombinant proteins correlates with protection of mice and monkeys to orthopoxvirus challenges. *Vaccine.* **2007**; 25:2787–99.
36. Golden JW, Josleyn MD, Hooper JW. Targeting the vaccinia virus L1 protein to the cell surface enhances production of neutralizing antibodies. *Vaccine.* **2008**; 26:3507–15.
37. Shinoda K, Wyatt LS, Irvine KR, Moss B. Engineering the vaccinia virus L1 protein for increased neutralizing antibody response after DNA immunization. *Virol J.* **2009**; 6:28.
38. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol.* **2003**; 171:4969–73.